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Protective effects of melatonin against oxidative stress in Fmr1 knockout mice: a therapeutic research model for the fragile X syndrome

Abstract: Fragile X syndrome is the most common form of inherited mental retardation. It is typically caused by a mutation of the Fragile X mental-retardation 1 (Fmr1) gene. To better understand the role of the Fmr1 gene and its gene product, the fragile X mental-retardation protein in central nervous system functions, an fmr1 knockout mouse that is deficient in the fragile X mental-retardation protein was bred. In the present study, fragile X mental retardation 1-knockout and wild-type mice are used to determine behaviour and oxidative stress alterations, including reduced glutathione, oxidized glutathione and thiobarbituric acid-reactive substances, before and after chronic treatment with melatonin or tianeptine. Reduced glutathione levels were depressed in the brain of fmr1-knockout mice and chronic melatonin treatment normalized the glutathione levels compared with the control group. Lipid peroxidation was elevated in brain and testes of fmr1-knockout mice and chronic melatonin treatment prevents lipid peroxidation in both tissues. Interestingly, chronic treatment with melatonin alleviated the altered parameters in the fmr1-knockout mice, including abnormal context-dependent exploratory and anxiety behaviours and learning abnormalities. Chronic treatment with tianeptine (a serotonin reuptake enhancer) did not normalize the behaviour in fmr1-knockout mice. The prevention of oxidative stress in the fragile X mouse model, by an antioxidant compound such as melatonin, emerges as a new and promising approach for further investigation on treatment trials for the disease.

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Introduction

Fragile X syndrome (FXS) is a rare inherited disorder, mostly characterized by moderate to severe mental retardation [1]. FXS affects approximately one in 4000 males and one in 8000 females in the general population [2]. Physical abnormalities include a long face with prominent ears, arched palate, hyperextensible joints, mitral valve prolapse and macroorchidism [3]. Hyperactivity, attention difficulties, anxiety and autism are symptoms of FXS [4]. The syndrome is caused by a mutation of the fragile X mental retardation 1 (fmr1) gene located on the X chromosome. The instability of CGG repeats in the fmr1 gene is the most frequent mutation and the absence of the fmr1 gene functional protein (FMRP) is responsible for the phenotype [5]. FMRP is an mRNA-binding protein that regulates local protein synthesis and mRNA transportation [6]. FMRP is highly expressed in neurones, and is associated with translating ribosomes in the cytoplasm, in dendrites and dendritic spines [7]. FMRP is densely expressed in the mouse adrenal medulla, without co-expression of the homologous proteins FXR1P and FXR2P; therefore, FMRP may have a specific function in this tissue [8].

The fmr1-knockout mouse (fmr1-KO) is considered to be a validated animal model for the experimental analysis of FXS [9]. It shows macroorchidism, abnormal dendritic spines with altered synaptic plasticity, mild learning impairment, increased susceptibility to seizures, enhanced responses to auditory stimuli, and hyperactivity with abnormal anxiety response [10, 11]. In general, the knockout mice exhibit more subtle behavioural phenotypes than those reported in the human syndrome [12].

Glucocorticoid receptor (GR) expression was reduced by 40% in the hippocampus of fmr1-KO mice [13]. The hippocampus is an important region of the limbic system, modulating hypothalamic–pituitary–adrenocortical (HPA) function, mood, learning and memory [14]. Taken together, findings from FXS patients and fmr1-KO mice suggest a defect in the negative feedback mechanism involved in recovery from stress-induced glucocorticoid release [15, 16]. Chronic stress is known to alter neuroendocrine, physiologic and behavioural parameters, affecting several brain regions [17]. Stress can contribute to neuronal degeneration in the brain, especially in the hippocampus [18]. In contrast to stress-induced catecholamine effects, that are more likely to be associated with emotionally laden memories, glucocorticoids are capable

of modulating hippocampal synaptic plasticity and changing dendritic structures [19].

There is a reported increase in extracellular glutamate levels in hippocampus and other brain regions after stress. Glutamate toxicity, enhanced by Ca^{2+} signals, may involve the participation of reactive oxygen species (ROS) and oxidative stress in brain damage. Release of excitatory neurotransmitters, such as glutamate, induces a cascade of reactions in the postsynaptic neurones, resulting in the formation of ROS. Because of their high chemical reactivity, levels of ROS, in excess of the normal needs of the cell, may indiscriminately damage structural and functional brain integrity [17].

A key player in the development of brain dysfunction is oxidative stress. Our previous studies in the fmr1-KO mouse model have linked increased generation of ROS, NADPH-oxidase activation and moderate increase in brain oxidative stress, as a pathological mouse model for the FXS [20, 21]. Oxidative stress markers were found to be increased in neurological diseases, ageing, and also in the FXS. For scavenging cytotoxic ROS, a major endogenous antioxidant system is present in cells. The brain contains almost no catalase and less glutathione peroxidase (GPx) and vitamin E, compared with the liver [22].

An endogenous antioxidant produced in the pineal gland, called melatonin (*N*-acetyl-5-methoxytryptamine), is found to be a powerful scavenger of ROS [23]. During oxidative stress, glutathione (GSH), one of the most important molecules in cellular defence against ROS is depleted, and this can be prevented with the use of melatonin [24]. Melatonin protects brain tissue against oxidative damage [25].

Tianeptine, a selective 5-HT reuptake enhancer with antidepressant effects in humans [26], has been reported to prevent stress-induced behavioural deficits [27]. It decreases the HPA axis response to stress [18], and protects the hippocampus against the deleterious effects of stress and glucocorticoids [19]. After acute or chronic treatment, tianeptine induces an increase in serotonin in rat and in humans and has been reported to be effective in treating autistic children [28].

In this study, we perform a treatment trial with both melatonin, a potent well-known antioxidant for the central nervous system (CNS), and tianeptine an anti-anxiety and antidepressant compound indicated to normalize behaviour and glucocorticoid plasma levels. To analyse the effectiveness of the chronic treatment to normalize oxidative stress, we performed free-radical, lipid and protein oxidation and antioxidant measurements; furthermore, behavioural studies and adrenal cortical hormones have been characterized in the fmr1-KO mice, in comparison with the proper WT-control groups.

Material and methods

Reagents

Melatonin was purchased from Sigma Aldrich (Sigma Chemical Co., St. Louis, MO, USA) and tianeptine was kindly provided by Servier Laboratories (Servier, Neuilly sur Seine, France).

Experimental animal model

The fmr1-knockout FVB-129 mouse strain animal model used for the experiments was obtained as a gift from B. Oostra (Erasmus University Rotterdam). They were kept as a mouse colony in the experimental animal house of the University of Malaga. All experimental protocols regarding the handling of experimental animals meet the guidelines of the Animal Welfare Committee of the University of Malaga and the European Communities Council Directive (86/609/EEC) and current Spanish legislation for the use and care of laboratory animals (BOE 252/34367-91, 2005). Mice were housed under controlled conditions of temperature and humidity, a 12-hr light/dark cycle with free access to standard food and water. We performed the experiments on 120 male mice aged 90–120 days. Male mice from different crossings (heterozygous wild-type/fmr1-knockout female and homozygous fmr1-knockout male) were randomized into experimental groups with four to six animals per cage (medium size) and handled daily.

Genotype analysis

Genotyping was performed using DNA extracted from mouse tail tissue. Briefly, the tissue was incubated overnight at 55°C in a buffer containing [10 μg/μL proteinase K; 20% sodium dodecyl sulphate; 10 mM Tris–HCl; 400 mM NaCl; and 2 mM ethylenediaminetetraacetic acid (EDTA)]. Polymerase chain reaction (PCR) was performed using Ready Mix Kit (Sigma Chemical Co.) and specific sets of primers described in the original article [9]. PCR reactions were carried out on a thermocycler (Mastercycler gradient; Eppendorf, Hamburg, Germany) and the products (knockout allele of 800 bp and wild-type allele of 465 bp) were visualized by electrophoresis using ethidium bromide in a 1.5% agarose gel.

Chronic treatments

Either vehicle, melatonin or tianeptine, was injected daily into 3-month-old mice for 30 days. All compounds were dissolved in ethanol and subsequently diluted in physiological saline (final ethanol content 0.01%) and administered intraperitoneally shortly after lights were on at 09:00 hours. An effective dosage of 10 mg/kg/day melatonin or tianeptine was used as previously described in the literature [29, 30]. Control groups were injected with vehicle (1 mL/kg/day of 0.01% ethanol in 0.9% NaCl).

Preparation of peritoneal macrophages

Peritoneal macrophages were isolated from mice and kept under standard conditions. The animals (30–32 g) were killed by cervical dislocation, and peritoneal macrophages were extracted by intra-peritoneal saline injection of 5 mL. The cells were pelleted by centrifugation, suspended in Hepes-buffered Krebs–Ringer solution (KR-Hepes), composed of 1.18 mM NaCl, 4.75 mM KCl, 1.18 mM H_2PO_4, 1.18 mM $MgSO_4$, 1.25 mM $CaCl_2$, 10 mM glucose and 25 mM Hepes (pH 7.4), and used immediately for experiments.

Intracellular H_2O_2 production

The production of ROS was analysed by 2,7-dichlorohydrofluorescein diacetate (DCFDA) as an indicator of the quantity of intracellular H_2O_2. Briefly, 2×10^6 (cells/mL) macrophages were incubated at 37°C in the dark in the presence of 2.5 μM DCFDA (dissolved in ethanol). The cells were then rinsed twice with KR-Hepes buffer, and the fluorescence intensity was measured at different times in a spectrofluorometer using excitation and emission wavelengths of 503 and 529 nm, respectively, with a Clarity Microplate Reader (Biotek, VT, USA). Intracellular ROS production was measured in brain slices after in vitro 1 mM melatonin incubation for 24 hr at 37°C and 5% CO_2. Protein concentration was determined in the cellular fractions analysed in this study by the Bradford method [31], and the final values were corrected and expressed as the percentage of fluorescence in control wells.

Brain dissection

Animals were killed by cervical dislocation. The brains were removed immediately at 4°C. For ex vivo experiments, the brain was sliced into 100-μm-thick slices and transferred onto plates containing sterile KR-HEPES buffer for ROS assays.

Measurement of total ROS production

Luminol-based luminescence correlated well with total ROS produced by the cells. As luminol can permeate freely through the cell membrane, the luminescence was an indication of the addition of intracellular plus extracellular ROS [32]. Briefly, brain slices were suspended in KR-Hepes, to which 15 μM luminol was added. The reaction was started by PMA-100 nM, and the chemiluminescence emission was recorded. Total ROS production was measured in brain slices after in vitro 1 mM melatonin incubation for 24 h at 37°C and 5% CO_2.

Protein oxidation assays

The formation of carbonyl compounds is actually the most general and widely used marker of severe protein oxidation both in vitro and in vivo [33]. We have followed the method described previously with slight modifications [34]. Samples of tissues were homogenated in phosphate buffer and then membrane and cytosol fractions were obtained by centrifugation at 25,000 g for 30 min. Both fractions were incubated in 10 mM 2,4-dinitrophenylhydrazine (DNPH) prepared in 2.5 M HCl for the test sample and 0.4 mL of 2.5 M HCl for the control samples. Then 0.5 mL of 20% TCA (w/v) was added to both tubes and the mixture left in ice for 10 min. The tubes were then centrifuged at 3500 g for 20 min to obtain the protein pellet. Finally, the precipitates were washed three times with 0.4 mL of ethanol:ethyl acetate (1:1, v/v) to remove unreacted DNPH and lipid remnants. The final protein pellet was dissolved in 0.2 mL of 6 M guanidine hydrochloride and incubated at 37°C for 10 min. Carbonyl content was determined by taking the spectra of the representative samples at 355–390 nm (VERSAmax Molecular Devices, Sunnyvale, CA, USA). The carbonyl content was calculated using an absorption coefficient (e) of 22,000/M/cm, and expressed as nmol/mg protein. Protein concentration was determined in the cellular fractions analysed in this work by the Bradford method [31].

Lipid peroxidation measurements

The tissue samples were homogenized with ice-cold buffer for the determination of thiobarbituric acid-reactive substances (TBARS). We determined lipid peroxidation under basal conditions in the enriched brain and testes membrane fractions, extracted as described previously [35]. The amount of TBARS produced was measured as the spectrophotometric absorbance of the supernatant at 532 nm (VERSAmax Molecular Devices). The absorbance measurements were calculated with a standard curve using (MDA) malondialdehyde-bisdiethyl-acetal (Sigma Chemical Co.). The final values were expressed as nmol of TBARS per mg of protein determined using the Bradford method [31].

Antioxidant measurement

Glutathione levels were determined in whole-brain extracts by spectrofluorometry [36]. A spectrofluorometric reading of the different samples was then obtained at an excitation wavelength of 350 nm and an emission wavelength of 440 nm using a fluorescence microplate reader (FLX800TBI; Bio-Tek Intruments USA). The results were compared against a standard curve of glutathione (Sigma Chemical Co.) and were expressed as mol GSH/g tissue and %GSSG (oxidized glutathione).

Hormonal assay

We assayed plasma corticosterone concentration using the commercially available ^{125}I radioimmunoassay kits from ICN Pharmaceuticals (Orangeburg, NY, USA).

Behavioural analysis

The habituation profile was characterized by the exposure to an open-field behavioural paradigm studying the response in novelty and familiarity (i.e. 24 hr later) both in basal conditions and after treatment. The animals were kept in the test room for 30 min prior to the start of the experiments.

The open-field and testing procedures were similar to those described previously [37]. Each experimental animal was placed in a dimly illuminated observation cage ($109 \times 49 \times 49$ cm) with a clear front pane. Activity in the open-field maze was tracked using a digital video camera coupled to the SMART software (PANLAB, Barcelona, Spain). The maze is virtually divided into two areas, centre and perimeter; we recorded the percentage of time spent in each area during 15-min periods as well as the number of crosses between areas and the total distance travelled.

The elevated plus-maze was based on that designed and validated by Lister [38]. Each mouse was placed in the

central square (5 × 5 cm) facing an open arm, and allowed to explore the maze for 5 min, during which time a video tracking system recorded its behaviour. The following variables were analysed: time spent in the open arms, closed arms and central square; open-arm entries and closed-arm entries (both the absolute number and as a percentage of total arm entries). The maze was thoroughly cleaned with a dry cloth between sessions. The anxiety response was characterized by the exposure to plus maze behavioural paradigm in the novelty condition.

The shuttle box was used to assess mouse fear-conditioning learning. On the first day the mice were placed individually in a clear methacrylate chamber (Med Associates, E. Fairfield, VT, USA) for fear-conditioning training [39]. A 30-s 85-dB tone [conditioned stimulus (CS)] was activated after a 2-min acclimation period in the chamber. Mice received a 2-s foot shock [unconditioned stimulus (US)] of 0.2 mA intensity during the last 2 s of the CS. The mice remained in the chamber for 30 s after the foot shock. Twenty-four hours later, the mice were returned to the chamber for a 5-min contextual test in the absence of the tone or foot shock. After a 1-hr waiting period, the mice were exposed to an alternate chamber (of different shape but the same area, with a black plastic floor and lemon-scented walls) for a 5-min cued test interval, in which the CS tone was presented during the final 3 min. Conditioning throughout training and testing was measured by assessing freezing behaviour. Mice were classified as frozen whenever complete absence of motion was evident (respiration excluded). The percentage of time spent frozen was calculated for each mouse in discrete time intervals, and these results were pooled and averaged for each group in each interval.

Statistical analysis

Data are expressed as mean ± standard error of the mean (S.E.M.) and the statistical significance of differences in mean values was assessed by Student's *t*-test or analysis of variance (ANOVA), as appropriate. All analyses were performed with the Statistical Package for Social Sciences (SPSS 10.5). A *P*-value of < 0.05 was considered significant.

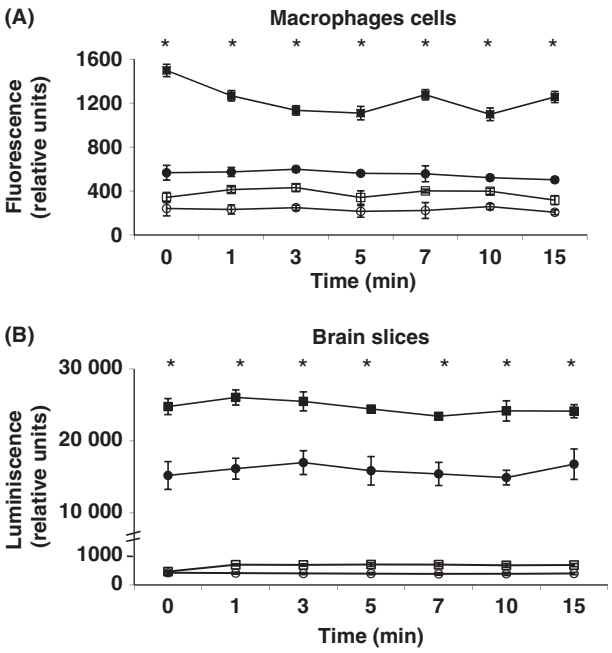

Fig. 1. The fmr1-KO mice displayed an increase in ROS production in macrophage cells and brain slices, compared to WT-control mice. (A) Intracellular free-radical production was measured in peritoneal macrophages from 4-month-old fmr1-KO and WT-control mice as described in *Material and Methods*. Under basal conditions, the fmr1-KO showed an increase in free-radical production compared with the WT-control group. In-vitro 1 mM melatonin treatment for 24 hr normalizes the macrophage free-radical overproduction to the normal WT-control level. (B) Total ROS production was measured in brain slices from fmr1-KO and WT-control mice as described in *Material and Methods*. Under basal conditions, Fmr1-KO showed an increased in total ROS production in comparison with the WT-control group. (C) After in vitro 1 mM melatonin treatment for 24 hr, the total ROS production in the fmr1-KO group was normalized to the WT-control level. Basal-WT data are indicated with (-●-), basal KO data are indicated with (-■-), Mel-WT data are indicated with (-○-) and Mel-KO data are indicated with (-□-). Data are mean ± S.E.M. of at least six to eight mice per group. Statistical significance of quantification was assessed by repeated-measure ANOVA, followed by Bonferroni post test. Values of $P < 0.05$ were considered significant.

Results

We designed a chronic treatment trial to assess the effectiveness of melatonin and tianeptine on the biochemical and behavioural characteristics of the Fragile X mouse model, and compared chronic treated groups with naïve and vehicle-treated control groups. The fmr1-KO mouse showed an increase in total ROS production measured in macrophage cells and brain slides in basal conditions (Fig. 1A,B). The in vitro 1 mM melatonin treatment for 24 hr normalized the free-radical production in macrophage cells (Fig. 1A) and brain slices (Fig. 1C).

The effectiveness of the melatonin treatment was analysed, measuring the carbonyl content of protein, a marker of protein oxidation. The total protein content was measured in brain and testicles, and no significant difference was detected between genotypes or treatments (data not shown). However, the measurement of carbonyl content in brain of both vehicle and naïve fmr1-KO groups showed a significant increase in this protein oxidation marker, which was reversed under chronic 10 mg/kg/day melatonin treatment (Fig. 2A). The carbonyl content in testes of both vehicle and naïve fmr1-KO groups showed a significant increase, and this increase was reversed under chronic 10 mg/kg/day melatonin treatment (Fig. 2B).

Thiobarbituric acid-reactive substances were measured as an index of lipid peroxidation in the plasma membrane fraction from brain and testicles obtained from mice of the fmr1-KO and WT-control groups treated chronically either with the vehicle or tested compound by intraperitoneal injection. TBARS levels in brain membranes were significantly higher in the fmr1-KO group compared with that in the WT-control group in naïve and vehicle conditions (Fig. 3A). The chronic administration of 10 mg/kg/day of melatonin normalized the TBARS levels in the fmr1-KO.

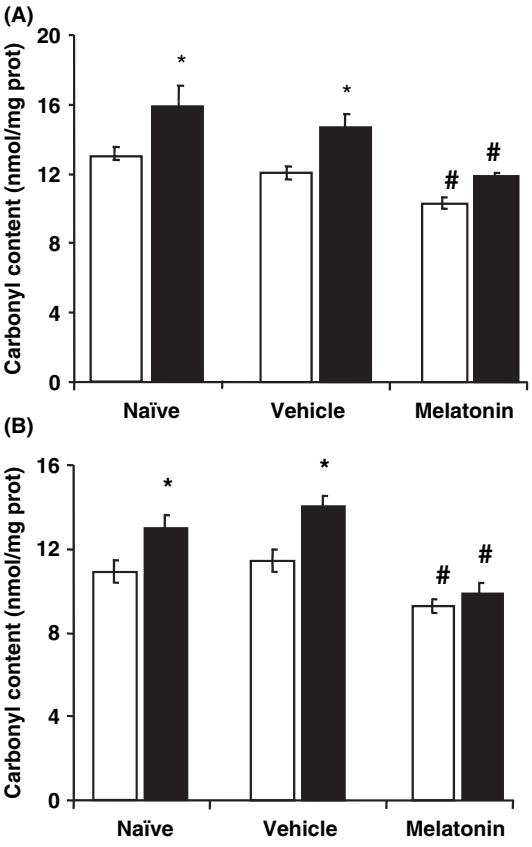

Fig. 2. Melatonin treatment reduced levels of carbonyl content of protein in brain and testicles from fmr1-KO mice. (A) Brain homogenates; (B) testicle homogenates. WT data are indicated with □, KO data are indicated with ■. Data presented are mean values ± S.E.M. Statistical significance of quantification was assessed by unpaired *t*-test. Values of $P < 0.05$ were considered significant. $^{*}P < 0.05$ Fmr1-KO versus WT-control; $^{\#}P < 0.05$ treated versus vehicle.

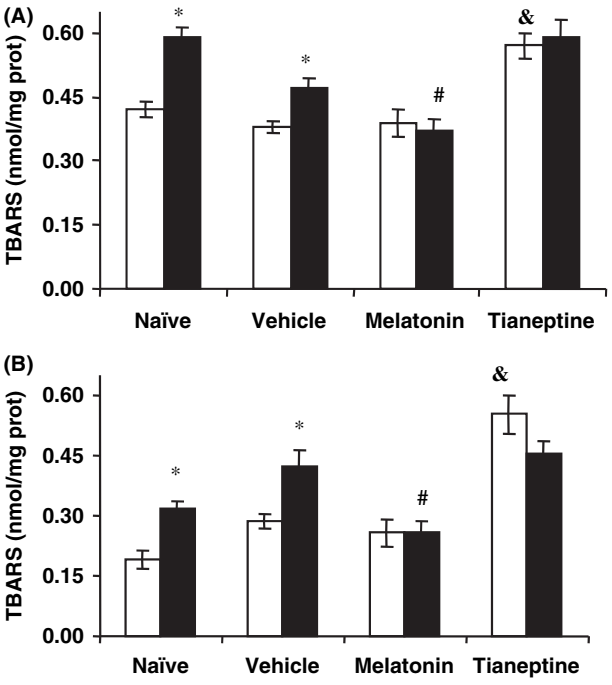

Fig. 3. Melatonin treatment reduced TBARS levels in brain and testicles from fmr1-KO mice. Determination of lipid peroxidation by quantifying TBARS by spectrophotometry in WT (n = 9) and Fmr1-KO (n = 8) mice after chronic treatment with melatonin and tianeptine in enriched brain (A) and (B) testes membrane fractions. Data presented are mean values ± S.E.M. WT data are indicated with □, KO data are indicated with ■. Statistical significance of quantification was assessed by unpaired *t*-test. Values of $P < 0.05$ were considered significant. $^{*}P < 0.05$ Fmr1-KO versus WT-control; $^{\#}P < 0.05$ treated-KO versus vehicle-KO, $^{\&}P < 0.05$ treated-WT versus vehicle-WT.

The TBARS levels were significantly increased in the brain of WT-control mice when tianeptine was administered chronically in comparison with that in WT-control groups under vehicle and naïve conditions (Fig. 3A).

In testicles of mice of the naïve fmr1-KO group, TBARS levels were significantly increased by 25% compared with naïve WT-controls and by 15% compared with vehicle WT-controls (Fig. 3B). Chronic treatment with 10 mg/kg/day melatonin normalizes testicular fmr1-KO TBARS levels to WT-control levels. Chronic tianeptine treatment significantly increases TBARS levels in both genotypes (Fig. 3B).

Glutathione is a key intracellular antioxidant, and it serves as a reductant to eliminate hydrogen peroxide and prevent lipid peroxidation. To assess whether the antioxidant properties of melatonin are suitable to maintain GSH homeostasis, the levels of reduced glutathione (GSH) and oxidized glutathione (GSSG) were measured in brain homogenates obtained from mice of the fmr1-KO group and WT group. GSH levels are decreased by 32% in brain homogenate of fmr1-KO mice compared with the vehicle WT-controls. Chronic treatment with melatonin normalizes GSH levels in the fmr1-KO group compared with the WT-control group. Chronic treatment with tianeptine slightly decreased brain GSH in both genotypes (Fig. 4A).

The GSSG level shows a 45% increase in the fmr1-KO mouse brain compared with the WT-control group in naïve and vehicle conditions. Chronic treatment with melatonin normalizes the altered GSSG measurements but treatment with tianeptine is unable to normalize GSSG levels in the fmr1-KO group. Moreover, a significant increase in the GSSG is observed in the WT-tianeptine group compared with that in the vehicle and naïve groups (Fig. 4B).

In order to characterize if the trial improves the abnormal behaviour observed in the Fragile X mouse model, such as hyperactivity and anxiety, two behavioural paradigms were performed in fmr1-KO and WT-control groups. The open field was performed in novelty and in familiarity conditions to study the habituation profile. The fmr1-KO group shows a significant increase in activity compared with the WT-control group in naïve and vehicle conditions, and can be observed in novelty (Fig. 5A) and familiarity conditions (Fig. 5B). A normalization of the locomotion activity was observed in the fmr1-KO group in comparison with the WT-control group after chronic administration of melatonin. In familiarity conditions, the fmr1-KO group showed an increase in locomotion compared with the WT-control group in naïve groups.

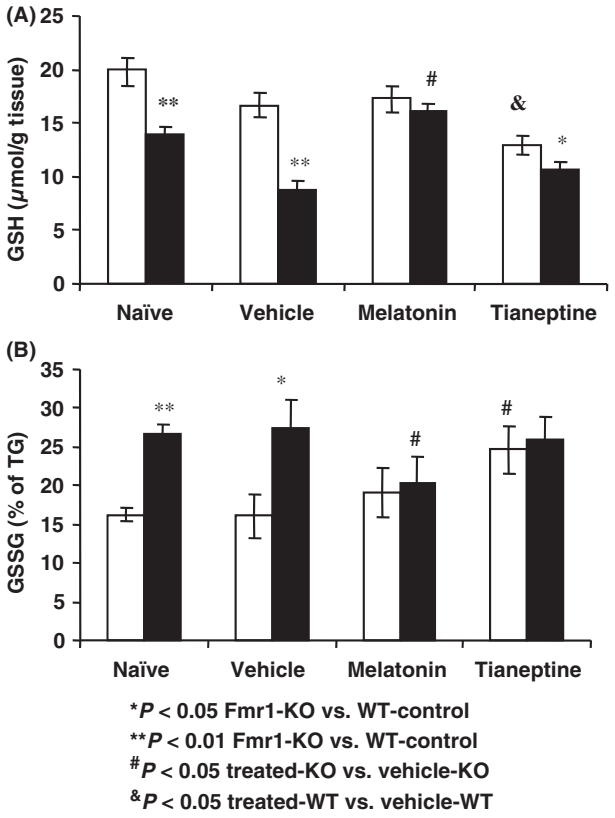

Fig. 4. Melatonin treatment enhanced GSH levels and reduced %GSSG in brain from fmr1-KO mice. Glutathione levels were determined in whole-brain extracts from Fmr1-KO mice (n = 10) and WT mice (n = 11) by spectrofluorometry and the results were compared against a standard curve of glutathione and were expressed as: (A) mol GSH/g tissue; (B) and %GSSG. Data are mean values ± S.E.M. WT data are indicated with □, KO data are indicated with ■. Values of $P < 0.05$ were considered significant. $^{*}P < 0.05$ Fmr1-KO versus WT-control; $^{**}P < 0.01$ Fmr1-KO versus WT-control; $^{\#}P < 0.05$ treated-KO versus vehicle-KO, $^{\&}P < 0.05$ treated-WT versus vehicle-WT.

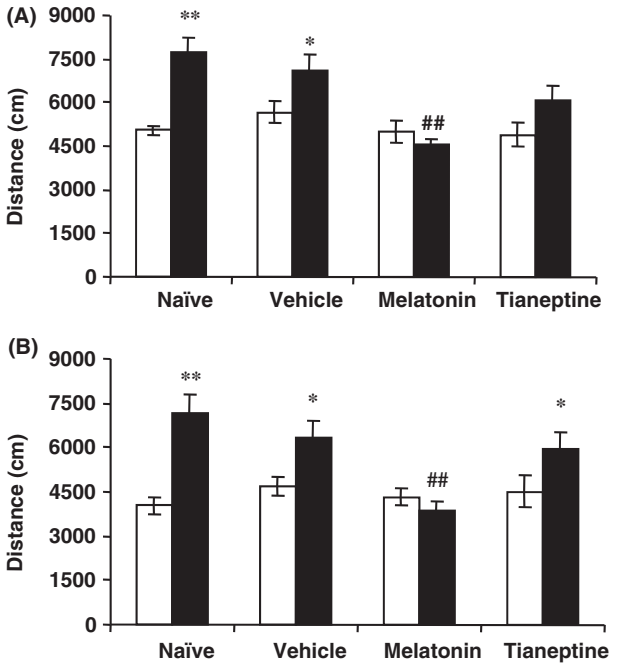

Fig. 5. fmr1-KO mice display hyperactivity in novelty and familiarity environments, and show an absence of the habituation profile. The activity of mice in the maze was tracked using a digital video camera coupled to the SMART software program. Locomotion distance during 15-min test in the open-field maze in (A) novelty and (B) familiarity environments of male fmr1-KO (n = 15) and WT (n = 16) in naïve condition and after chronic treatment to male fmr1-KO (n = 10) and WT (n = 11) mice. Data presented are mean values ± S.E.M. WT data are indicated with □, KO data are indicated with ■. Values of $P < 0.05$ were considered significant. $^{*}P < 0.05$ Fmr1-KO versus WT-control; $^{**}P < 0.01$ Fmr1-KO versus WT-control; $^{\#}P < 0.05$ treated-KO versus vehicle-KO.

The WT-control group exhibited a significant decrease in the exploration 24 hr after the first exposure to the open field (5000 cm versus 4100 cm; $P < 0.05$). This attenuated exploration has been previously described in mice [38]. Moreover, the fmr1-KO group did not show the attenuated response exhibited in familiarity by the WT-control group [7500 cm versus 7400 cm]. Fmr1-KO mice under chronic treatment with tianeptine showed a significant increase in activity in familiarity conditions. In the elevated plus maze, the animals are exposed to an approach/avoidance conflict and a reduced open-arm exploration is considered to reflect increased anxiety.

In the total distance travelled in the elevated plus maze test (Fig. 6A), no differences were found in both genotypes in naïve and vehicle groups; however, the total distance was reduced in the treated groups in comparison with the naïve and vehicle groups. The percentage of time spent in the open arms (Fig. 6B) under naïve and vehicle conditions was increased in fmr1-KO mice in comparison with the WT-control mice, reflecting lower anxiety or anxiolysis in the Fragile X mouse model. In addition, no difference was found between genotypes under chronic melatonin treatment. Tianeptine treatment did not produce normalization of the anxiety response between the fmr1-KO and WT groups. The total number of entries showed no significant difference between genotypes in naïve and vehicle groups (Fig. 6C). A significant decrease in the number of entries was observed in the melatonin-treated group in comparison with the vehicle group.

In order to further identify these behaviour context-dependent responses, we characterized the adrenal response to stress. The glucocorticoid plasma level was reduced in the fmr1-KO naive group. Immediately after acute stress, corticosterone levels were significantly increased in plasma of the fmr1-KO mouse compared with that of the WT-group. Chronic treatment with melatonin normalized the corticosterone level in the fmr1-KO group. Tianeptine treatment did not normalize corticosterone levels in the fmr1-KO group (Fig. 7).

To study the effectiveness of chronic melatonin treatment in hippocampal/amygdala learning deficits we analysed learning fear conditioning. In our experimental conditions, the fmr1-KO mouse showed no difference in freezing response compared in contextual experiments of the testing session (Fig. 8A). The fmr1-KO mouse showed less freezing

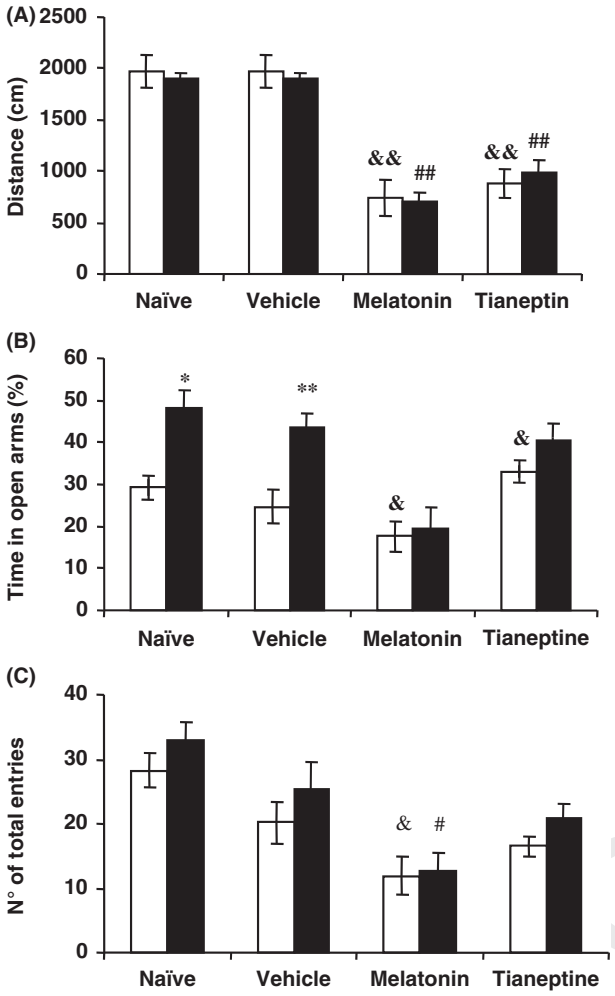

Fig. 6. Effect of chronic treatment (10 mg/kg/day) with Melatonin and Tianeptine in the behaviour of male Fmr1-KO mouse in the elevate plus-maze. (A) Fmr1-KO mice displayed normal activity in the elevated plus-maze. After chronic treatment, activity was reduced in males of both genotypes, the fmr1-KO (n = 10) and WT (n = 11) mice. (B) Fmr1-KO mice displayed anxiolysis in the elevated plus-maze. The percentage of time spent in the open arms was elevated in the Fmr1-KO in the elevated plus-maze. Chronic Melatonin treatment normalizes the anxiety response in the elevated plus-maze. (C) The number of total arm entries was unchanged in the vehicle and naïve conditions, and reduced after chronic melatonin treatment in both genotypes. Data presented are mean values ± S.E.M. WT data are indicated with □, KO data are indicated with ■. Values of $P < 0.05$ were considered significant. $^{*}P < 0.05$ Fmr1-KO versus WT-control; $^{**}P < 0.01$ Fmr1-KO versus WT-control; $^{\#}P < 0.05$ treated-KO versus vehicle-KO; $^{\#\#}P < 0.01$ treated-KO versus vehicle-KO; $^{\&}P < 0.05$ treated-WT versus vehicle-WT; $^{\&\&}P < 0.01$ treated-WT versus vehicle-WT.

response than the WT-control group in cued experiments during the testing session in naïve conditions (Fig. 8B). Mice that were subjected to a chronic 10 mg/kg/day melatonin treatment showed no differences in the cued experiments during the testing session (Fig. 8B), as the melatonin treatment normalized the fear conditioning learning.

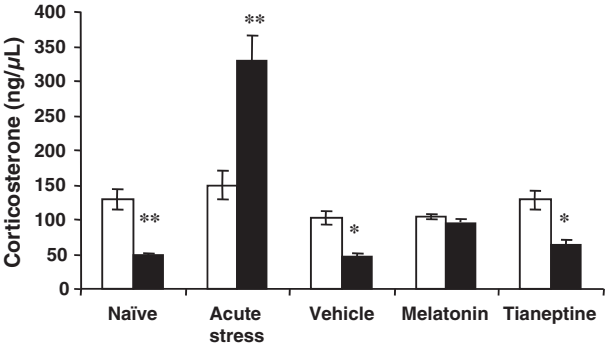

Fig. 7. Melatonin treatment normalizes corticosterone levels in the fmr1-KO mice. Corticosterone levels (ng/μL) were measured in serum using a commercial competitive RIA kit. Serum levels are decreased in male fmr1-KO mice (n = 10) versus WT mice (n = 11) in naïve and vehicle conditions, elevated in acute stress conditions. Chronic 10 mg/kg melatonin treatment normalizes the fmr1-KO serum corticosterone levels. Chronic tianeptine treatment failed to normalize the levels. Data presented are mean values ± S.E.M. WT data are indicated with □, KO data are indicated with ■. Values of $P < 0.05$ were considered significant. $^{*}P < 0.05$ Fmr1-KO versus WT-control; $^{**}P < 0.01$ Fmr1-KO versus WT-control.

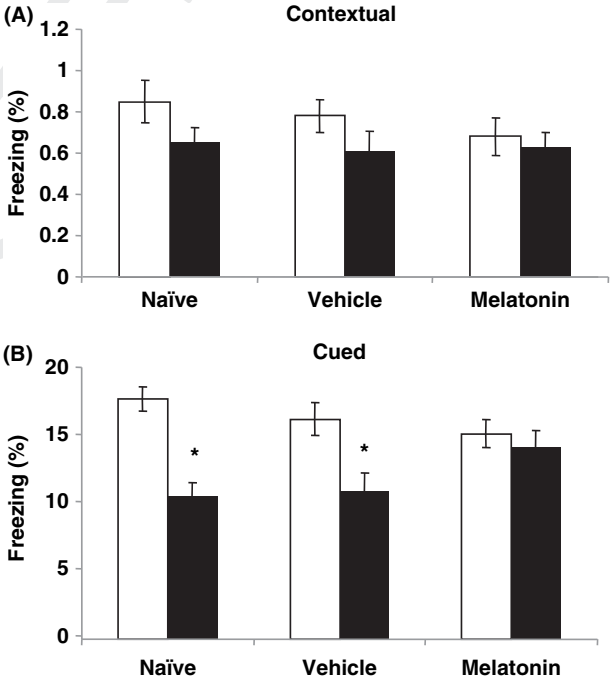

Fig. 8. The absence of FMRP protein impairs cued fear conditioning and chronic 10 mg/kg Melatonin treatment improves learning deficits observed in the fmr1-KO mouse model. (A) 24 hr after a training session, the mouse is tested for contextual fear conditioning. In our experimental conditions there are not differences in the contextual fear conditioning comparing genotypes. (B) fmr1-KO mouse groups under naïve and vehicle conditions displayed significantly decreased levels of freezing for cued fear conditioning, in comparison with the WT-control group. Chronic 10 mg/kg/day Melatonin treatment normalized the measured learning abnormalities indicating hippocampal/amygdala memory deficits observed in the fmr1-KO mouse. Data are means ± S.E.M. of at least 10–12 mice per group. WT data are indicated with □, KO data are indicated with ■. Values of $P < 0.05$ were considered significant. $^{*}P < 0.05$ Fmr1-KO versus WT-control.

Discussion

The present study provides experimental evidence that chronically administered melatonin normalizes several abnormalities observed in the fmr1-KO mouse, including biochemical hallmarks, such as free-radical production in macrophage cells and brain slices, as well as carbonyl content in proteins and lipid peroxidation. Additionally, it also normalizes reduced glutathione levels in the brain and testicles of fmr1-KO mice. The treatment also controls corticosterone plasma levels, locomotion (hyperactivity), anxiety responses and fear learning deficits. Chronic treatment with tianeptine failed to normalize the fmr1-KO mice. Therefore, all these findings together indicate that chronic treatment with a therapeutic dose of melatonin protected the fmr1-KO mouse from the oxidative stress pathology in brain and testes, the most affected organs described in the FXS; it also reversed several behavioural and learning deficits.

This study was designed to determine the effectiveness of exogenously administered 10 mg/kg/day melatonin; this dose is comparable to the dose previously used in mice [29]. In humans, exogenous melatonin, at a dose of up to 0.2 mg/kg, is generally used for anaesthesia premedication [40]. As an antioxidant in amyotrophic lateral sclerosis patients, 5 mg/kg melatonin was administered up to 4 months [41]. The exogenous melatonin dosage of 10 mg/kg in mice is reasonably conservative, which is about 1 mg/kg when extrapolated to the human dose according to the species difference between mice and humans. A pharmacological dose of 10 mg/kg/day tianeptine was used and it was comparable with the dose previously used in mice [30].

The biochemical abnormalities in free-radical production and in oxidative stress parameters were previously described by our group [20, 21], and behavioural alterations have also been identified in the fmr1-KO mouse [9, 10]. Previously published experiments have also found that melatonin significantly ameliorated cognitive impairment and increased antioxidative enzymes in mouse. [29]. The clear protective effects of melatonin against oxidative stress and also behavioural and learning deficits observed in our study are consistent with previously published reports [42].

Tianeptine is a tricyclic compound with a dibenzothiazepine structure, acting as a selective serotonin reuptake enhancer [26]. At 10 mg/kg it reduced non-social activity and social investigation and increased mouse immobility. Tianeptine exhibits an anxiogenic-like behavioural profile in male mice tested in the plus-maze [43]. It shows a good therapeutic efficacy in patients classified as 'anxious-depressed' [44]. In our model, the tianeptine treatment was unsuccessful to normalize the Fmr1-KO mouse.

Brain tissue is highly vulnerable to free-radical damage because of its high oxygen utilization, high concentrations of polyunsaturated fatty acids and also low concentrations of cytosolic antioxidants [19]. Lipid peroxidation, as well as oxidative damage to proteins and DNA, has been found in age- and stress-associated functional deficits in many organs including the brain [45, 46]. Melatonin functions as free-radical scavenger and is highly protective in the brain against oxidative damage [24, 25]. Melatonin is a very efficient scavenger of highly toxic OH [47] and also neutralizes ROS which harm essential neuronal molecules such as peroxynitrite anion (ONOO) [48] and nitric oxide (NO) [49]. In addition to these scavenging actions, melatonin stimulates several antioxidative enzymes such as superoxide dismutase (SOD), GSH peroxidase (GPx) and GSH reductase which increase its efficiency as an antioxidant [23]. Furthermore, it increases the expression of mRNA encoding GPx and SOD in the rat cerebral cortex [50, 51]. Melatonin has an important role in the maintenance of the GSH antioxidant, as it has been shown to stimulate γ-glutamylcysteine synthetase, the rate-limiting enzyme in GSH synthesis, as well as recycle GSH within the cell. A significant decline in total glutathione levels was observed in aged mice, which was reversed by chronic melatonin treatment [25]; even in our experiments, the glutathione level increased after chronic treatment with melatonin. We have proposed in our previously published study that these oxidative markers are important hallmarks in the fragile X pathophysiology [20, 21] and they can be reversed by a chronic melatonin treatment as demonstrated in the present study.

Lipid peroxidation is a damaging process in the brain caused by free-radical activity over the high concentration of polyunsaturated fatty acids (PUFA) in the CNS. Melatonin is effective in reducing lipid peroxidation [48]; in this study too, chronic melatonin treatment has been proven to reduce the TBARS in the brain and testes of the Fmr1-KO mouse. A preventive effect of melatonin on ethanol-induced lipid peroxidation in rat testes was described previously [52].

Exogenous melatonin prevents the behavioural abnormalities associated with oxidative stress, such as motor activity and diminished recall ability on the second day of exposure to the test paradigm [53]. Fmr1-KO mice show hyperactivity and have a mild spatial learning impairment in the Morris water maze [9]; they also exhibit increased exploratory activity and decreased anxiety-like responses [54], and chronic melatonin treatment reversed these two behavioural abnormalities. The 10 mg/kg/day melatonin treatment significantly ameliorated learning and memory performance in the fmr1-KO model. It was proposed previously that chronic melatonin treatment reverses cognitive deficits in diabetic, aged and ethanol-intoxicated mice, which is associated with its antioxidant property [42, 55]. Moreover, the daily melatonin treatment in stressed mice prevented several chronic stress-induced disturbances, including reduction in spontaneous locomotor activity [56]. Melatonin treatment prevents mild cognitive impairment in patients suffering from Alzheimer's disease (AD) or other dementia disorders [57].

The HPA axis is an important component of the biological stress response system; it is adaptive and prepares the individual for dealing with the source of the stress; however, chronic elevations of corticoadrenal secretion can lead to medical problems [19] and can have adverse effects in brain areas expressing glucocorticoid receptors such as the hippocampus, amygdala and cortex that interfere with behaviour, learning and memory [58]. Interactions between melatonin secretion and L-HPA axis are well established [59], and chronic melatonin treatment considerably alters the affinity of glucocorticoid receptors in the brain [60].

Neuroendocrine studies of individuals with FXS have documented a number of hypothalamic–pituitary abnormalities, including precocious puberty and elevated gonadotrophin levels [61]. Boys with FXS have greater variability in total sleep time and difficulty in sleep maintenance. Elevated nocturnal and daytime melatonin levels were reported in salivary samples of fragile X patients [62]. Abnormal melatonin secretion may thus be responsible for some of the phenotypic features of this disorder. Our model showed a significant improvement of the Fmr1-KO mouse abnormalities after chronic melatonin treatment. Even though the melatonin abnormalities were described in FXS, the results from melatonin trials have not been published for FXS.

The neuroendocrine study showed that in comparison with their siblings, children with fragile X, especially males, had elevated basal cortisol levels during the day and before bedtime, and they had a greater cortisol response to challenges [15]. Glucocorticoids released in response to stress are known to potently modulate memory, in both rodents and humans, and it increases with the open-arm exposure in the elevated plus maze [63]. Our experimental model showed an increase in the corticosterone plasma level in response to acute stress, and melatonin treatment normalizes corticoadrenal secretion, this may be related to behavioural and biochemical improvements.

Secondary melatonin metabolites have antioxidative protective properties, safeguarding mitochondrial electron flux. It has been demonstrated in many experimental systems that melatonin metabolites are particularly neuroprotective [64]. We have not analysed melatonin metabolites in this study but it is possible that part of the observed benefits are induced by their positive actions.

Melatonin also inhibits the phosphorylation of the p^{47}phox subunit of NADPH oxidase via a PI3K/Akt-dependent signalling pathway, and impairs the assembly of NADPH oxidase to produce free radicals in the cell membrane [65]. We have previously described that NADPH oxidase is activated in the brain of the Fmr1-KO mouse [20], and its downregulation of the enzyme by melatonin treatment can be hypothesized as a possible molecular pathway involved in the reduction of free-radical production observed in the Fmr1-KO mouse tissues after chronic treatment.

Finally, the present study indicates for the first time that melatonin treatment ameliorates the phenotypic alterations of the Fmr1-KO mouse, such as free-radical production, oxidative stress markers, corticoadrenal hormonal secretion, and behavioural and learning deficits. It may represent a new experimental approach for the therapeutic research of the FXS, a rare inherited disorder with reduced therapeutic options.

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